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14. ABSTRACT Mechanisms establishing and controlling tissue polarity are central both to normal breast function and breast cancer. Molecular studies have revealed key proteins that mediate and maintain cell- and tissue- polarity; however, the fundamental biophysical processes within a single cell must undergo multiple rounds of mitosis to assemble into polarized tissue-like structures and \ that are the fundamentals of loss of tissue architecture are hardly understood. We placed single non-malignant cells in 3-D laminin-rich (IrECM) cultures to observe what % of the cells could form 3-D acinar structures. Using high-resolution real time imaging, we identified a morphogenic behavior where mammary epithelial cells undergo organized multiple rotations to establish an acinus (a sphere with hollow lumen). We named this movement: Quasi-coherent Angular motion (CAMo). Here we are investigating the role of CAMo in normal development, aging and breast cancer progression.					
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Introduction:

In order to understand what molecular and biophysical mechanisms drive cancer progression, it is imperative to understand also the mechanisms that are required to establish and maintain normal tissue. Molecular studies have revealed key proteins that mediate and maintain cell and tissue polarity. However the biophysical processes that a single cell must go through as it multiplies through several rounds of mitosis to assemble into a polarized and functional tissue are not well understood. Even less understood is what biophysical properties lead to disruption of tissue architecture, which is a precursor and driving factor behind breast cancer.

To answer these questions, we placed single non-malignant or malignant isogenic human mammary epithelial cells (HMEC) into 3D cultures of a gel made of laminin-rich extracellular matrix (lrECM). Then we used high-resolution real time imaging to observe the morphogenic progression of acinar formation. Acini are spheres with a hollow lumen and are the functional unit of the human breast. We found that non-malignant HMECs undergo organized multiple rotations (in either direction) to establish an acinus with correct apical-basal polarity. We named this spinning movement coherent angular motion (CAMo) (Tanner et al, 2012 PNAS 109:1973). Interestingly, breast tumor cells do not undergo CAMo but move in a more randomized fashion. However, when the tumor cells were treated with agents that caused ‘reversion’ of the malignant phenotype, which allows the tumor cells to ‘behave’ as non-malignant cells, CAMo was restored. Restoration of CAMo was correlated with the formation of correctly polarized colonies resembling the non-malignant cell colonies in 3D cultures of lrECM. Furthermore, when we disrupted the interaction of non-malignant breast epithelial cells with agents that block correct cell-cell contact, such as interference with E-cadherin, the non-malignant cells did not undergo CAMo and formed disorganized colonies that were not spheres. These original studies published in Tanner, et al., 2012, suggest that CAMo is an important component for development and maintenance of normal tissue architecture and loss of CAMo may be a biophysical driving force behind the loss of correct tissue architecture that precedes breast cancer. To help address these questions we proposed to (I) To determine the occurrence/nature of CAMo by other mammary cell types in 3D cultures and *in vivo* (II) To functionally compare and contrast coherent angular motion as a developmental morphogenetic program required in the establishment and the maintenance of the mammary gland as a function of age.

Key Words: mammary gland, breast cancer, three-dimensional cell culture, morphogenesis, coherent angular motion (CAMo), live cell imaging, confocal microscopy

Overall Project Summary: During this first year of funding we have concentrated our work to address studies proposed in Task 1 particularly focused on Subtask 1.1a and 1.1c

Task 1: To determine the occurrence/nature of CAMo by other mammary cell types in 3D cultures and *in vivo*.

Subtask 1.1a: Real time live cell imaging using confocal microscopy will be used to image cellular movement of myoepithelial cells, ‘progenitor cells’, fibroblasts and fat cells embedded in different ECM. This will include the creation of stable cell lines that are fluorescently tagged. CAMo was originally identified using the non-malignant human breast epithelial cell lines (S1 cells) from the HMT3522 breast cancer progression series and the MCF10A cell line grown in 3D cultures of lrECM. However, the mammary gland is composed of other interacting cell types, including myoepithelial cells. These cells are most important as they lie adjacent to the milk producing luminal epithelial cells and we, and others, have evidence that these cells are absolutely crucial in proper function of the luminal cells (Gudjonsson et al, JCS, 2002). During lactation, myoepithelial cells provide the physical forces to squeeze the milk from the lumen of the acinus down through the ducts to the nipple. Myoepithelial cells also produce the basement membrane proteins that surround the acinus *in vivo* and they are thought to protect the luminal cell population from becoming tumorigenic. However, the biochemical and biophysical mechanisms of how myoepithelial cells protect the breast from tumor progression or how they are lost is not completely understood.

In order to address questions surrounding the role of myoepithelial cells, we asked the question: do they behave as epithelial cells and undergo CAMo. When we placed myoepithelial cells in 3D cultures of lrECM and tracking their movement by confocal microscopy using a vital dye, we found that they do undergo CAMo through the first few cell divisions. These preliminary experiments are exciting as they are important steps in understanding behavior of normal myoepithelial cells using live cell imaging in physiologically relevant cultures. We have just begun these studies, as there was initial concern from the DoD that some overlap existed between this funding and funding of a pending R37 merit award from the NIH, where we had planned to do many studies with human cells under the umbrella of this funding. However, since that time, all merit awards from the NIH were discontinued including this one already awarded! As a result this apparent overlap got resolved. Thus, the experiments designed to investigate CAMo more deeply using human cells lines will not be funded by NIH and we plan to use human cell lines as well as mouse cell lines and tissues to investigate the importance of CAMo in the breast. Future plans are to examine CAMo in other human breast cells such as fibroblasts and fat cells and if time permits examine co-cultures of epithelial and myoepithelial cells in lrECM as well as other ECM components such as collagen I.

Subtask 1.1c: Mouse mammary gland anlage will be excised and embedded in ECM gels, such as collagen I and lrECM. Using real time live cell imaging the nature of the cellular motion will be monitored and analyzed. If anlage are not viable, then single cells derived from mouse mammary glands will be used. Physiologically relevant 3D cultures of mouse cells are very useful in the investigation of mammary gland development and cancer progression. This is particularly true for development, as the majority of mammary gland development occurs post-natal and mammary glands can be harvested at different ages/stages of development, pregnancy and involution. Furthermore, organotypic 3D cultures of mouse mammary glands are more economical than strict in vivo studies as one mouse can provide tissue to perform several experiments. For developmental studies, we also utilize a functional mouse mammary cell line from Balb/c (EpH4 cells). When grown in 3D culture of lrECM, EpH4 cells form organized acinar-like colonies with a hollow lumen and amazingly they can be induced to secrete milk (ie β -casein) into the lumen of the colony.

In studies this year to address subtask 1.1c we have used EpH4 cells as well as mammary organoids to determine (1) do mouse mammary epithelial cells undergo CAMo like human breast epithelial cells and (2) does CAMo actually occur in the developing mammary tissue. We used a vital dye (Cell Tracker™) and live cell confocal microscopy to image EpH4 cells (Figure 1) and organoids from Balb/c mice in 3D lrECM (Figure 2 upper panels). We also used tissue from a transgenic C57BK/6 mouse line where histone H2B is linked with GFP (H2B-GFP) to observe the cells in the organoid culture (Figure 2 lower panels). EpH4 cells or organoids were placed in culture imaged and analyzed for movement. So far we found that EpH4 cells, when in the acinar structures, rotate together. We did not observe CAMo per se. However, the EpH4 cells tend to clump together when plated in 3D culture. This is a technical issue and syringing the cells seems to break these clumps apart allowing for single cell plating. When future experiments are done with the EpH4 cells this syringing technique will be used to obtain cultures that start with single cells. With the mouse organoids random movement is observed early, but once the structure reaches completion and begins to form the end buds (pseudo acini) there is rotation within the buds that looks very much like CAMo. These preliminary results suggest that rotation is involved in the formation of spherical structure that makes an organized acini both in culture and in vivo. For the in vivo situation, there may be some sorting involved prior to the final acinar-like structure formation. Further studies are needed and will be conducted using the H2B-GFP mouse organoids to obtain better quantification. We have attempted to perform the organoid studies using collagen I gels. However, we have had difficulties keeping the organoids alive in this matrix. It is possible that the laser used in the confocal microscope is too harsh under the conditions used for the collagen I gels and ‘fine tuning’ of conditions will allow us to observe the development of branched mammary structures in collagen I.

Task 2. To functionally compare and contrast coherent angular motion as a developmental morphogenetic program required in the establishment and the maintenance of the mammary gland as a function of age.

In the next year of funding, we plan to use mouse mammary organoids from different ages of mice as well as human mammary epithelial and myoepithelial cells collected from women at different ages to examine whether CAMo changes with age. If we find that cells from older individuals do not undergo CAMo, this will provide a basis for looking at mechanisms that led to breast cancer as women age.

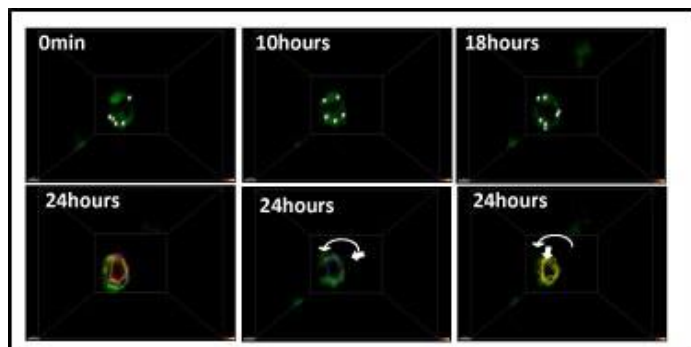


Figure 1. Mouse mammary cells form acinar-like structures that collectively rotate in 3D cultures of lrECM. EpH4 cells were grown in 3D lrECM and visualized by live cell imaging using a CellTracker™ dye (green). The nuclei of cells in the acinar structure have been labeled with a white dot. The time course is shown in hours and the 24 hr time point was quantified. Two tracks of the rotation have been singled out in the last 2 panels and the direction of the rotation is shown with a white arrow.

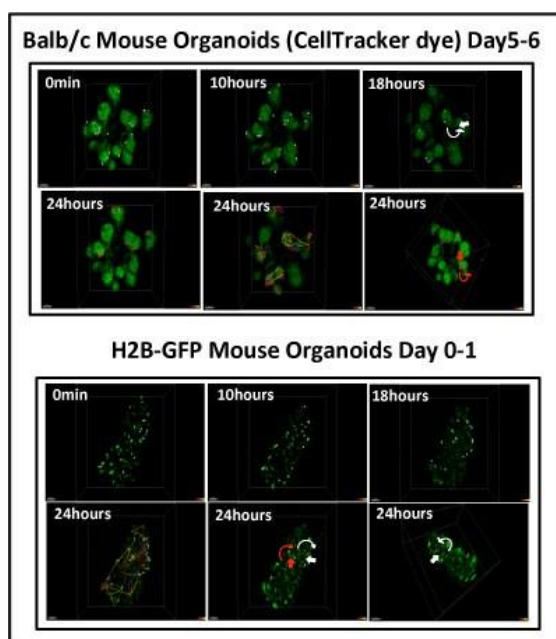


Figure 2. Cells inside developing mammary organoids appear to display CAMo in physiologically relevant 3D cultures of lrECM. 3D organoid culture of Balb/c mice (upper panel) or transgenic H2B-GFP mice (lower panel) were evaluated for CAMo using live cell imaging. Time course of images is shown. The images for the 24 hr time point were analyzed for cellular movement. Near the end of the developing organoid bud, cells were observed undergoing CAMo-like movement. The rotation of cells at 24 hr is shown in the last to panels of each organoid by white and red arrows. Further experiments are needed to evaluate this movement further to ensure it is indeed CAMo.

It should be noted that the initial plan was to continue this work much more closely with Dr. Kandice Tanner who now has her own laboratory at the NCI. We offered to send the graduate student who had done some work to her laboratory to perform some of the experiments. But Dr. Tanner explained to me that this is not an advisable course because NCI is not as open of an entity now as it used to be. In addition she explained that she has been advised strongly not to continue to do her research with the postdoctoral mentor and must do independent work. Whereas we still have one project that we will hopefully continue to do together, I am quite sympathetic to her situation and have not pressed her any further and asked her to let us know when she is ready for the collaboration and at this point the next action must come from her laboratory. Since there was no way to send someone to Dr. Tanner's laboratory, we are doing the work essentially in Berkeley instead of sending someone to Dr. Tanner's laboratory our new postdoctoral fellow Dr. Clair Robertson who has just arrived is a mechanical bioengineer who is very interested in continuing aspects of the project that were in this application. Dr. Robertson is an expert in imaging and image analysis and in fact trained at University of California, Irvine, where Dr. Tanner also trained. Of course we are offering to do this collaboratively with Dr. Tanner, in a manner that will not stop the progress of her own papers and the establishment of her independent laboratory (i.e. it will basically all will be done here).

Key Research Accomplishments:

- Imaging and analyzing mouse mammary organoids from Balb/c and H2B-GFP transgenic mice in IrECM and collagen I gels
- Imaging human myoepithelial cells

Conclusions:

During this first year of funding, we have build on our original observations regarding the importance of CAMo in the establishment of functional mammary gland. We will continue to use the organotypic cultures to define the role of CAMo in developing mammary gland and determine what happens with aging as proposed in the original SOW.

There are no Publications or Presentations; Inventions; or Reportable Outcomes to report for this first year of this funding.

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